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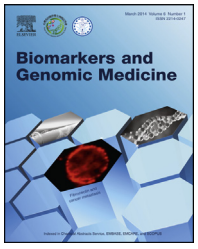

ORIGINAL ARTICLE

Effect of oxygen tension on proliferation and characteristics of Wharton's jelly-derived mesenchymal stem cells

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Abstract Mesenchymal stem cells (MSCs) from Wharton's jelly have a higher proliferation rate and self-renewal capacity than adult tissue-derived MSCs. A low oxygen level or hypoxic condition is prevalent in the microenvironment of the stem cells in the early stages of development. Hypoxia can influence proliferation and differentiation of various stem/precursor cell populations. This research was conducted: to determine the proliferation rate and characteristics of human MSCs from Wharton's jelly in hypoxic and normoxic condition; to evaluate their character after MSCs are incubated in hypoxic and normoxic environment using surface markers including CD105, CD73, CD14, CD19, CD34, CD45, and HLA-II; and to evaluate the proliferation rate and number of MSCs at many passages using the trypan blue method. The hypoxic and normoxic microenvironment showed significant differences in the proliferation rate and population doubling time, but and there were no differences in surface markers.

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Introduction

Stem cells are currently used in clinical applications[1] and can be obtained from embryonic and extraembryonic tissues and adult organs. Stem cells have the ability to prolong self-renewal and differentiate into mature cells of various lineages, which makes them important cell sources for tissue engineering applications.[1,2] Clinical therapies require a large number of cells, so many strategies are used to improve the quality and quantity of stem cells.[3]

The clinical therapeutic strategy uses mesenchymal stem cells (MSCs) as cellular vehicles for the targeted delivery and local production of biologic agents in many diseases.[4] MSCs were originally isolated from the bone marrow (BM). BM-derived MSCs (BM-MSCs) are nonhematopoietic precursor cells, and are capable of contributing to the maintenance and regeneration of connective tissues through engraftment.[5] However, BM-MSCs have: limited cell numbers, a risk of loss of stem properties, chromosomal changes, and problems of contamination, painful isolation procedure, low MSC characteristics, multipotent differentiation potential, and proliferation efficiency of BM-MSCs decline with increasing age.[6–8] MSCs are hypoimmunogenic and have the ability to promote regeneration and functional recovery in disease and injury, which involves immunomodulation effects. MSCs are good candidates for cell transplantations and allogeneic applications.[9]

MSCs are able to differentiate to a variety of specialized mesenchymal tissues including bone, cartilage, muscle, marrow stroma, tendon, ligament, fat, and connective tissue.[10] MSCs have been isolated from different compartments of the umbilical cord (cord blood, umbilical cord matrix, and the perivascular region), adult peripheral blood,[11,12] adipose tissue,[13] lung,[14] heart,[15] trabecular bone, and dental pulp,[16] and also from a variety of fetal tissues, such as the spleen, lung, pancreas, kidneys, and amniotic fluid during midgestation.[17] MSCs can be isolated from Wharton's jelly (WJ), the embryonic mucous connective tissue lying between the amniotic epithelium and the umbilical vessels. Wharton's jelly-derived MSC or WJ-MSCs have a higher proliferation rate and self-renewal capacity than adult tissue-derived MSCs.[18,19]

Oxygen concentration is an important component of the stem cell niche, where it plays an important role in maintaining the proliferation and plasticity of stem cells.[1,20] The oxygen concentration has been investigated extensively.[21] Several stem cell populations cultivated under hypoxic condition resulted in enhanced proliferation.[1] Physiological oxygen tension of 5% instead of 2% was found to improve a mouse embryonic stem cell line by reducing oxidative stress.[22] Hypoxic conditions have been shown to maintain the pluripotency and minimize spontaneous differentiation of human embryonic stem cells.[20] Mammalian cells exposed to hypoxic conditions express a variety of target genes controlled by hypoxia inducible factor 1 to overcome hypoxic stress.[23] Hypoxic conditions can increase the number of hematopoietic stem cells.[24]

The objective of this research was to evaluate the effect hypoxic environment can have on the proliferation and surface marker character of WJ-MSCs. The results of this research may be useful from a clinical point of view, as WJ-MSCs are used for cell therapy to repair tissue injuries, the MSCs can encounter severe low oxygen tension.

Materials and methods

Isolation and cultivation of WJ-MSCs

Fresh human umbilical cords (UC; $n = 5$) were collected from women aged 25–40 years after normal vaginal delivery, with informed consent using the guidelines approved by the Institutional Ethics Committee at the Stem Cell and Cancer Institute, Jakarta, Indonesia and from the Institutional Ethics Committee collaboration between Maranatha Christian University, Bandung, Indonesia and Immanuel Hospital Bandung, Bandung, Indonesia.

MSCs from WJ of UC, were isolated as previously described.[3,25] UC was washed by phosphate buffer saline (0.9% w/v sodium chloride) and cut into very small pieces, approximately 1–2 mm, then UC was cut longitudinally, and plated on tissue culture plastic plates. The explants were cultured in MEMα with 2 mM GlutaMAX (Invitrogen, Carlsbad, CA, USA), supplemented with 20% fetal bovine serum (FBS; Invitrogen) and penicillin–streptomycin–amphotericin B (100 U/mL, 100 μg/mL, and 0.25 μg/mL; Invitrogen). Cultures were incubated in a humidified atmosphere with 5% CO_2 at 37 °C for 3 weeks after explantation, when fibroblast-like adherent cells were expected to migrate from the tissue fragments, the adherent cells and tissue fragments were detached using trypLE–EDTA solution (TrypLE Express; Invitrogen) followed by washing with basal medium to remove the trypLE–EDTA. The cells were harvested and replated at a density 8×10^3 cells/cm^2 when cells reached 80–90% confluence. WJ-MSCs were cultured in 95% air (21% O_2)/5% CO_2 for normoxic and hypoxic (5% and 2.5% oxygen). Hypoxia was achieved using a tri-gas incubator (CO_2 incubators with additional process controls; BINDER GmbH, Tuttlingen, Germany) with internal O_2 and N_2 tank changer for connecting to separate gas tanks.

Cell proliferation analysis

The effect of hypoxic and normoxic incubation towards the cells proliferation was determined as follows. Cells were counted and passaged at a confluence of 80%. Briefly, cultured cells were dissociated using trypsin, incubated for 3 minutes at 37 °C, harvested and washed using MEMα + 20% FBS followed by centrifugation at $300 \times g$, for 4 minutes. The cell pellet was resuspended with trypan blue solution (0.4% in PBS, 1:1 dilution with culture medium) for 3 minutes. The number of dead cells (retaining the dye) was counted with a hemocytometer and expressed as a percentage of the total viable cell number. The experiments were performed in triplicate.

At each passage, the population doubling (PD) was determined using the formula:

$$PD = [\log_{10}(NH) - \log_{10}(NI)]/\log_{10} \quad (1)$$

where NI is the inoculum cell number and NH the cell harvest number. PD for each passage was calculated and added to the PD of the previous passages in order to generate cumulative PD data. The PD time was obtained by the formula:

Table 1 Cumulative cell number, population doubling (PD), PD time, and cumulative PD of mesenchymal stem cells in normoxic tension.

Passage	Cumulative cell number	PD	PD time, h	Cumulative PD
P1	$1.82 \times 10^6 \pm 1.51 \times 10^5$ [a]	1.92 ± 0.12[b]	1.57 ± 0.09[a]	1.57 ± 0.09[a]
P2	$6.90 \times 10^6 \pm 4.50 \times 10^5$ [a]	1.94 ± 0.08[b]	1.55 ± 0.06[a]	3.12 ± 0.08[b]
P3	$3.52 \times 10^7 \pm 1.36 \times 10^6$ [a]	2.34 ± 0.04[c]	1.71 ± 0.03[a]	4.83 ± 0.05[c]
P4	$1.71 \times 10^8 \pm 2.85 \times 10^7$ [a]	2.27 ± 0.19[c]	1.77 ± 0.15[a]	6.60 ± 0.19[d]
P5	$5.65 \times 10^8 \pm 5.78 \times 10^7$ [a,b]	1.73 ± 0.13[b]	1.74 ± 0.14[a]	8.34 ± 0.12[e]
P6	$1.95 \times 10^9 \pm 2.97 \times 10^8$ [b]	1.78 ± 0.08[b]	2.25 ± 0.10[b]	10.59 ± 0.22[f]
P7	$4.39 \times 10^9 \pm 4.57 \times 10^8$ [c]	1.18 ± 0.10[a]	3.41 ± 0.28[c]	14.00 ± 0.21[g]
P8	$1.53 \times 10^{10} \pm 1.37 \times 10^{9}$[d]	1.80 ± 0.08[b]	3.34 ± 0.14[c]	17.34 ± 0.32[h]

Data are presented as mean ± standard deviation. Different letters in the same column (among passage) are significant at $p < 0.05$ (Tukey's honestly significant differences *post hoc* test).

$$\text{PD time} = t/\text{PD (in hours)}, \quad \text{where } t = \text{time} \tag{2}$$

At the same time, a growth curve of WJ-MSCs from two different conditions was started. Cells were seeded at 200/cm^2 in 6-well plates. Every passage (3 days culture) for eight passages, cells from one well were harvested and counted.

Detection of MSCs markers using fluorescence activated cell sorting (FACS)

The WJ-MSCs were evaluated using surface marker detection at Passage 4 (P4) and P8 to confirm the effect of oxygen concentration (hypoxia and normoxia) on MSC characterization, WJ-MSCs at 80% confluence were harvested and dissociated with trypsin-EDTA and centrifuged at 300 × *g* for 10 minutes. The pellet was resuspended with PBS + 2% FBS, and cells were counted with a hemocytometer. Between 100 cells and 200 cells in 25 μL PBS were introduced into FACS (BD FACSCalibur™) tubes. Antibody was then added to each FACS tube: isotype mIgG2a-PE, CD105-PE, HLA class II-PE; isotype mIgG1-PE, CD73-PE, CD19-PE; isotype mIgG1-FITC, CD 34-FITC, CD45-FITC, CD14-FITC, followed by incubation at 4 °C for 15 minutes. The cells were analyzed by flow cytometry with a FACSCalibur 3 argon laser 488 nm (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest Pro Acquisition on the BD FACStation™ Software. The experiments and measurement of surface marker were performed in triplicate.

Results

Effect of oxygen tension on stemcell proliferation

The results of evaluating the effect of oxygen tension (hypoxia, normoxia) on WJ-MSCs proliferation, cumulative cell number, PD, PD time, and cumulative PD for each passage up to P8 are in Tables 1—3. Based on these data, PD time including normoxic, hypoxic (O_2 2.5%; O_2 5%) P1—P5 had the same PD time and old passage (P6—P8) had higher a PD time than young passage on normoxic and hypoxic tension. In order to compare the cumulative cell number, PD and PDT among incubation (normoxic and hypoxic tension) at every passage, the data were analyzed using Tukey's honestly significant differences *post hoc* test (Tables 4 and 5). Hypoxia 2.5% had a higher cumulative cell number compared to normoxia and hypoxia 5% at P4, P5, P6, and P8. The cells were seeded at 480.000 cells for all treatment (normoxia, hypoxia 5%, hypoxia 2.5%) and at all passages (P1—P8). Table 5 shows that PD of hypoxia 2.5% at P3, P4, P7, and P8 was higher than normoxia and hypoxia 5% O_2 PD rate. PD time hypoxia 2.5% was lower than normoxia and hypoxia 5% at P7 and P8.

Effect of oxygen tension on phenotype

Human MSCs surface marker are suggested to be positive for CD73 and CD105 and negative for CD14, CD19, CD34, and

Table 2 Cumulative cell number, population doubling (PD), PD time, and cumulative PD of mesenchymal stem cells in hypoxic tension (5% O_2).

Passage	Cumulative cell number	PD rate	PD time, h	Cumulative PD
P1	$1.80 \times 10^6 \pm 1.20 \times 10^5$ [a]	1.90 ± 0.13[b,c]	1.59 ± 0.10[a]	1.59 ± 0.10[a]
P2	$6.58 \times 10^6 \pm 1.30 \times 10^6$ [a]	1.86 ± 0.19[b,c]	1.62 ± 0.18[a]	3.21 ± 0.26[b]
P3	$3.33 \times 10^7 \pm 7.80 \times 10^6$ [a]	2.33 ± 0.08[c]	1.72 ± 0.06[a]	4.93 ± 0.31[c]
P4	$1.66 \times 10^8 \pm 1.76 \times 10^7$ [a]	2.34 ± 0.37[c]	1.74 ± 0.26[a]	6.67 ± 0.14[d]
P5	$5.20 \times 10^8 \pm 8.69 \times 10^7$ [a]	1.64 ± 0.11[a,b]	1.83 ± 0.13[a]	8.50 ± 0.23[e]
P6	$1.80 \times 10^9 \pm 4.64 \times 10^7$ [a]	1.77 ± 0.16[a,b]	2.27 ± 0.19[b]	10.77 ± 0.41[f]
P7	$4.60 \times 10^9 \pm 1.3 \times 10^9$ [a]	1.35 ± 0.05[a]	2.98 ± 0.12[c]	13.75 ± 0.47[g]
P8	$1.84 \times 10^{10} \pm 5.12 \times 10^{9}$[b]	2.01 ± 0.00[b,c]	2.99 ± 0.01[c]	16.73 ± 0.47[h]

Data are presented as mean ± standard deviation. Different letters in the same column (among passage) are significant at $p < 0.05$ (Tukey's honestly significant differences *post hoc* test).

Table 3 Cumulative cell number, population doubling PD, PD time, and cumulative PD of mesenchymal stem cells in hypoxic tension (2.5% O_2).

Passage	Cumulative cell number	PD	PD time, h	Cumulative PD
P1	$1.88 \times 10^6 \pm 1.20 \times 10^5$ [a]	1.97 ± 0.09^a	1.53 ± 0.07^a	1.53 ± 0.07^a
P2	$7.20 \times 10^6 \pm 3.40 \times 10^5$ [a]	1.94 ± 0.04^a	1.55 ± 0.04^a	3.07 ± 0.05^b
P3	$4.36 \times 10^7 \pm 3.14 \times 10^6$ [a,b]	2.59 ± 0.04^a	1.54 ± 0.03^a	4.62 ± 0.07^c
P4	$2.64 \times 10^8 \pm 2.50 \times 10^7$ [a,b]	2.60 ± 0.04^a	1.54 ± 0.03^a	6.15 ± 0.10^d
P5	$9.74 \times 10^8 \pm 1.04 \times 10^7$ [a,b]	1.88 ± 0.07^a	1.60 ± 0.06^a	7.75 ± 0.11^e
P6	$3.78 \times 10^9 \pm 7.05 \times 10^8$ [a,b]	1.94 ± 0.16^b	2.07 ± 0.17^b	9.82 ± 0.25^f
P7	$1.31 \times 10^{10} \pm 1.93 \times 10^{9b}$	1.80 ± 0.17^c	2.23 ± 0.22^b	12.05 ± 0.19^g
P8	$6.48 \times 10^{10} \pm 1.2 \times 10^{10}$ [c]	2.30 ± 0.09^c	2.62 ± 0.10^c	14.67 ± 0.28^h

Data are presented as mean ± standard deviation. Different letters in the same column (among passage) are significant at $p < 0.05$ (Tukey's honestly significant differences *post hoc* test).

Table 4 Cumulative cell number among normoxic and hypoxic tension.

Passage	Cumulative cell number		
	Normoxia	Hypoxia 5%	Hypoxia 2.5%
P1	$1.82 \times 10^6 \pm 1.51 \times 10^5$ [a]	$1.80 \times 10^6 \pm 1.20 \times 10^5$ [a]	$1.88 \times 10^6 \pm 1.20 \times 10^5$ [a]
P2	$6.90 \times 10^6 \pm 4.50 \times 10^5$ [a]	$6.58 \times 10^6 \pm 1.30 \times 10^6$ [a]	$7.20 \times 10^6 \pm 3.40 \times 10^5$ [a]
P3	$3.52 \times 10^7 \pm 1.36 \times 10^6$ [a]	$3.33 \times 10^7 \pm 7.80 \times 10^6$ [a]	$4.36 \times 10^7 \pm 3.14 \times 10^6$ [a,b]
P4	$1.71 \times 10^8 \pm 2.85 \times 10^7$ [a]	$1.66 \times 10^8 \pm 1.76 \times 10^7$ [a]	$2.64 \times 10^8 \pm 2.50 \times 10^7$ [b]
P5	$5.65 \times 10^8 \pm 5.78 \times 10^7$ [a]	$5.20 \times 10^8 \pm 8.69 \times 10^7$ [a]	$9.74 \times 10^8 \pm 1.04 \times 10^7$ [b]
P6	$1.95 \times 10^9 \pm 2.97 \times 10^8$ [a]	$1.80 \times 10^9 \pm 4.64 \times 10^7$ [a]	$3.78 \times 10^9 \pm 7.05 \times 10^8$ [b]
P7	$4.39 \times 10^9 \pm 4.57 \times 10^8$ [a]	$4.60 \times 10^9 \pm 1.3 \times 10^9$ [a]	$1.31 \times 10^{10} \pm 1.93 \times 10^9$ [b]
P8	$1.53 \times 10^{10} \pm 1.37 \times 10^{9a}$	$1.84 \times 10^{10} \pm 5.12 \times 10^{9a}$	$6.48 \times 10^{10} \pm 1.2 \times 10^{10}$ [b]

Data are presented as mean ± standard deviation. Different letters in the same row (among normoxic and hypoxic) are significant at $p < 0.05$ (Tukey's honestly significant differences *post hoc* test).

CD45.[26] The effect of oxygen tension on the surface marker of WJ-MSCs can be seen in Table 6. Table 6 shows that the surface marker of WJ-MSCs on hypoxia and normoxia, both P4 and P8, were not significantly different ($p > 0.05$).

Discussion

It has been reported that reduced oxygen tension enhances proliferation of some cell types, for example, hypoxia (1–5 % oxygen) enhanced the self-renewal of hematopoietic stem cells and murine embryonic stem cells in several previous studies. Moreover, when rat BM-MSCs were cultured in reduced oxygen condition, and the proliferation of MSC was increased and had a greater number of colonies.[27–31]

In the current study, the increased hypoxic (O_2 2.5%) condition was the best microenvironment for stem cells proliferation compared to normoxic and hypoxic (O_2 5%) for cells at a high passage (P7, P8). This result was consistent with previous reports that MSCs maintain viability when cultured in 2%–5% O_2, and increase their proliferation rate after an initial lag phase.[32] Hypoxic preconditioning of MSCs

Table 5 Population doubling PD, PD time among normoxic and hypoxic tension.

Passage	PD			PD time, h		
	Normoxia	Hypoxia 5%	Hypoxia 2.5%	Normoxia	Hypoxia 5%	Hypoxia 2.5%
P1	1.92 ± 0.12^a	1.90 ± 0.13^a	1.97 ± 0.09^a	1.57 ± 0.09^a	1.59 ± 0.10^a	1.53 ± 0.07^a
P2	1.94 ± 0.08^a	1.86 ± 0.19^a	1.94 ± 0.04^a	1.55 ± 0.06^a	1.62 ± 0.18^a	1.55 ± 0.04^a
P3	2.34 ± 0.04^a	2.33 ± 0.08^a	2.59 ± 0.04^b	1.71 ± 0.03^b	1.72 ± 0.06^b	1.54 ± 0.03^a
P4	2.27 ± 0.19^a	2.34 ± 0.37^a	2.60 ± 0.04^b	1.77 ± 0.15^a	1.74 ± 0.26^a	1.54 ± 0.03^a
P5	1.73 ± 0.13^a	1.64 ± 0.11^a	1.88 ± 0.07^a	1.74 ± 0.14^a	1.83 ± 0.13^a	1.60 ± 0.06^a
P6	1.78 ± 0.08^a	1.77 ± 0.16^a	1.94 ± 0.16^a	2.25 ± 0.10^a	2.27 ± 0.19^a	2.07 ± 0.17^a
P7	1.18 ± 0.10^a	1.35 ± 0.05^a	1.80 ± 0.17^b	3.41 ± 0.29^b	2.98 ± 0.12^b	2.23 ± 0.22^a
P8	1.80 ± 0.08^a	2.01 ± 0.00^a	2.30 ± 0.09^b	3.34 ± 0.14^c	2.99 ± 0.01^b	2.62 ± 0.10^a

Data are presented as mean ± standard deviation of PD and PD time. Different letters in the same row (among normoxia and hypoxia of PD, PDT) are significant at $p < 0.05$ (Tukey's honestly significant differences *post hoc* test).

Table 6 Surface markers of mesenchymal stem cells under normoxic and hypoxic tension.

Passage	CD34	CD45	CD14	CD105	CD73	CD19	HLA-II
P4							
Normoxia	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	94.28 ± 0.86	98.13 ± 0.53	−0.85 ± 0.61	−3.59 ± 0.50
Hypoxia 5%	0.00 ± 0.00	0.00 ± 0.01	0.01 ± 0.02	93.49 ± 2.64	98.35 ± 0.86	−0.54 ± 0.19	−2.22 ± 2.10
Hypoxia 2.5%	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	96.39 ± 2.86	97.02 ± 1.00	−0.28 ± 0.27	−2.80 ± 1.16
P8							
Normoxia	0.00 ± 0.00	0.00 ± 0.01	0.01 ± 0.01	94.87 ± 2.57	97.99 ± 0.92	−0.94 ± 0.64	−3.80 ± 1.96
Hypoxia 5%	−0.01 ± 0.00	0.00 ± 0.01	−0.01 ± 0.02	96.21 ± 2.64	99.16 ± 0.86	−0.21 ± 0.19	−2.68 ± 2.10
Hypoxia 2.5%	0.00 ± 0.01	0.00 ± 0.01	0.00 ± 0.01	95.85 ± 0.84	97.35 ± 1.01	−0.13 ± 0.08	−1.68 ± 1.26

Data are presented as mean ± standard deviation of surface markers of mesenchymal stem cells. The treatments were triplicate. P4 = Passage 4; P8 = Passage 8.

in 0.5% oxygen for 24 hours increased expression of pro-survival and proangiogenic factors including hypoxia-inducible factor 1, angiopoietin-1, and vascular endothelial growth factor and its receptor. Cell death of hypoxic stem cells and caspase-3 activation in these cells were significantly lower compared with normoxic stem cells both *in vitro* and *in vivo*.[33] Hypoxic conditions enhance cell amplification, and culturing under hypoxia could be an alternative approach without the need for extra additives to stimulate primary culture and further expansion, yielding a sufficient supply of cells and avoiding multiple passages.[34] Low-oxygen tension is an important component of the stem-cell microenvironment (the stem-cell niche) and provides signals conducive to maintenance of stem-cell function.[35] Compared with the normoxic condition, hypoxia enhances proliferation with an approximately six- to seven-fold higher expansion of adipose tissue-derived stromal cells over 6 weeks.[36] Hypoxia provides a favorable culture condition to promote proliferation of MSCs.[37] The long-term (1 month) effect of human MSC culture in hypoxic tension (2% O_2) showed improved survival and increased adipocytic and osteogenic differentiation capacity.[32] The MSC culture under hypoxic conditions was associated with the induction of hypoxia-inducing factor-α and an elevated expression of energy metabolism-associated genes including *glucose transporter 1* (*GLUT-1*), *lactate dehydrogenase* (*LDH*), and *pyruvate dehydrogenase kinase 1* (*PDK1*).[38] High concentrations of oxygen can cause oxidative stress via production of reactive oxygen species—free radicals that can damage lipids, proteins, and DNA, altering cell metabolism.[39] Moderate hypoxia may lower intracellular reactive oxygen species generation and accumulation and thereby increase the metabolic efficiency.[40]

The flow cytometric analysis (Table 6) showed that oxygen level and passage did not affect the MSC's character. The surface markers expression are positive for CD 105 and CD 73 (more than 95%) and negative for CD 14, CD 19, CD 34, CD 45 and HLA-II (less than 2%). CD45 is a pan-leukocyte marker; CD34 marks primitive hematopoietic progenitors and endothelial cells; CD14 is prominently expressed on monocytes and macrophages, the most likely hematopoietic cells to be found in an MSC culture; CD19 is a marker of B cells that may also adhere to MSCs in culture; and HLA-II-DR molecules are not expressed on MSCs.[41] Table 6 shows that surface markers in hypoxic condition were not significantly different when compared to normoxic. However, this result was not consistent with previous results that showed CD90 expression reduced in BMSCs harvested under hypoxia may be associated with improved chondrogenesis,[42] hypoxic culture for expansion of adipose tissue-derived stromal cells, and maintenance of their undifferentiated state.[36]

In conclusion, hypoxic 2.5% O_2 yield the highest proliferation, and the lowest PD and PD time. Oxygen level does not affect surface markers of WJ-MSCs at P4 or P8

Conflicts of interest

All contributing authors declare no conflicts of interest.

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